Chapter 7

Summary, General Discussion and Future Perspectives
SUMMARY OF FINDINGS IN THIS THESIS

The ageing process contributes to immune system dysfunction making the elderly population more prone to suffer from cancer and autoimmune diseases including vasculitic disorders such as anti-neutrophil cytoplasmic autoantibody (ANCA) associated vasculitis (AAV) and giant cell arteritis (GCA). Characterization and understanding of alterations of the ageing immune system may clarify the mechanisms by which ageing-related disorders develop. Therefore, this thesis aimed to explore such alterations focusing on aberrant expression of immune checkpoint molecules on the surface of immune cells and its functional consequences in health and disease.

More specifically, this thesis aimed to first study the impact of age and sex on immune checkpoint (IC) molecule expression by circulating immune cells of healthy individuals. As a next step, this thesis interrogated the contribution of changes in IC molecule expression to the pathogenesis of age-related vasculitides AAV and GCA by zooming in on immune cell subsets important in disease pathogenesis such as neutrophils and CD4+ T cells. In chapter 2, expression levels of surface IC (i.e. VISTA, PD-1, CD40L, ICOS and CTLA-4) on circulating immune cells in fresh blood samples from healthy young (aged 20-31) and old donors (aged 54-86) were analyzed and compared not only between young and old but also between men and women. The kinetics of IC expression on circulating T cells was analyzed after stimulation in vitro and compared between the different age and sex groups. We showed that PD-1 expression by circulating CD4+ T cells is affected by both age and gender. PD-1 expression frequencies within memory CD4+ T cells are decreased upon ageing and appear to be especially lowered in elderly females when compared to elderly males. Given the importance of T and B intercellular communication in the ageing immune system, we investigated IC molecules involved in T-B cell interaction (i.e CD40L and ICOS) and how these are modulated by age and sex. We showed that CD40L is increased in CD4+ and CD8+ T cells of elderly donors compared to younger individuals. In addition, there was an age-associated decline of ICOS by CD8+ T cells but not CD4+ T cells. The kinetics of IC expression revealed differences in magnitude between CD4+ and CD8+ T cells but did not seem to be affected by age and sex. Overall, we found that IC molecule expression by T cells is influenced by both age and sex. The decrease in frequencies of PD-1+ CD4+ memory T cells in elderly women only, warrants further research towards optimization of IC therapy and safety to prevent the development of immune related adverse events (irAEs).
To illustrate the importance of IC molecules in the development of vasculitides, we present a case report from our hospital in chapter 3 describing a melanoma patient treated with immune checkpoint inhibitors (ICI) who subsequently developed GCA. This case report demonstrates that ICI therapy may amplify cellular responses in susceptible individuals which can cause irAEs, in this case large-vessel vasculitis. In my thesis, I did not only focus on the more conventional IC molecules but I also investigated the role of VISTA, a recently described negative IC molecule which upon ligation suppresses T cell activation (1). Interestingly, mice genetically engineered to lack VISTA expression develop an age-related pro-inflammatory phenotype characterized by spontaneous T-cell activation (2). This observation implies that aberrant VISTA expression or function may contribute to pathogenic T cell mediated immune responses in age associated autoimmune disorders such as GPA and GCA. In chapter 4, we compared the frequencies of circulating VISTA positive leukocytes between GPA patients in remission and healthy controls. We found increased frequencies of VISTA expressing leukocytes in GPA patients compared to healthy individuals. Although the functional consequences of this observation remain to be elucidated, our results show that neutrophils express higher levels of VISTA following priming with TNF-α in vitro. In addition, preliminary results from co-cultures of autologous CD4+ T cells and neutrophils showed that unprimed neutrophils from GPA patients exert a higher suppressive effect on CD4+ T cells than those of HC. Possible explanations for this observation could be that neutrophils of GPA patients have already undergone in vivo TNF-α-priming or that this suppressive effect is due to the increased frequencies of VISTA positive neutrophils in the circulation of GPA patients in remission. Clearly, additional knowledge on VISTA in health and disease is needed to better understand the consequences of its increased expression on leukocytes in GPA patients.

CD4+ T cells are considered major players in GCA pathogenesis. In order to investigate the possible added contribution of VISTA to the dysregulation of CD4+ T cell responses in GCA, we first determined the expression of VISTA and other IC molecules on circulating monocytes and CD4+ T cells of GCA patients and infiltrating immune cells in temporal artery biopsies of GCA patients in chapter 5. Our data showed higher numbers of VISTA+, PD-1+ and PD-L1+ infiltrating cells in temporal artery biopsies of GCA patients suggesting a futile attempt to decrease immune activation and prevention of further damage. Since especially Th1 and Th17 cells are deemed important in the pathogenesis of GCA, we also determined the effect of VISTA-Ig engagement on CD4+ T cell subset lineage differentiation in vitro. Our results revealed a functional role of VISTA in GCA pathogenesis by showing that decreased expression of VISTA may facilitate development of pathogenic Th1 and
Th17 cells in GCA. In addition, we found that VISTA favors T follicular helper (Tfh) cell differentiation. The role of Tfh cells in ageing and in GCA remains to be further studied.

The analysis of the expression and dynamics of different IC molecules on the surface of several immune cells resulted in complex high-dimensional datasets which are not easy to analyze and interpret by traditional analysis methods. In chapter 6, we used a relatively new computational tool namely the t-distributed stochastic neighbor embedding (t-SNE) algorithm to visualize high-dimensional datasets (3). tSNE analysis enabled visualization of immune checkpoint expression on immune cells of our cross-sectional and follow-up GCA patients. Interestingly, tSNE analysis also enabled the identification of rare cell subpopulations which would have been unnoticed when using traditional manual gating. More specifically, we identified distinct immune populations in active GCA patients that were not present in GCA patients in remission or in healthy controls. Further detailed investigation of these rare sub-populations could perhaps provide clues to disease mechanisms. This study should be seen as a first step in the application of advanced computational analyses methods in the interpretation and visualization of multidimensional flow cytometry data in GCA.

IMPLICATIONS OF FINDINGS AND FUTURE PERSPECTIVES
The research described in this thesis revealed a functional role of VISTA in GCA pathogenesis and provided some clues regarding the involvement of this negative IC molecule in GPA. Clearly, additional research is warranted on the mechanism by which VISTA exerts its suppressive effects. Moreover, I encourage further research addressing the following questions:

Should future autoimmunity studies be stratified according to age and gender?
The female population is predominantly affected by autoimmune diseases (4,5). Although the underlying mechanisms are still not clear, research indicates that differences in levels of sex hormones may contribute to the higher incidence of autoimmune diseases such as SLE, Sjögren’s syndrome and rheumatoid arthritis in females (4,5). Estrogen levels decrease after menopause and the low estrogen levels can induce the production of pro-inflammatory cytokines such as TNF-α and IL-1β (5,6) making females more prone to develop age-related autoimmune disorders such as GCA. Other factors such as reproductive function, pregnancy and epigenetic influences could also explain female gender predisposition to autoimmunity (4,5).
Although there is a less clear association between age and the development of autoimmunity for some diseases like SLE, evidence shows that the immune system becomes more susceptible to develop (auto) immune-related disorders during ageing such as vasculitis. The latter may be a consequence of chronic low-grade inflammation in addition to the shifting balance from anti-inflammatory protective factors towards pro-inflammatory damaging factors. Despite the increase in knowledge on the immuno-pathology of age-related diseases, we are not yet able to prevent those diseases and clearly further research is warranted to identify targetable features induced by ageing (7).

This thesis demonstrates that IC molecule expression by healthy T cells is influenced by both age and sex. In addition, we showed that healthy elderly females have decreased frequencies of PD-1+ CD4+ T cells. Moreover, we showed that IC molecules involved in T-B cell interaction and autoantibody production such as CD40/CD40L and ICOS/ICOSL were also affected by age. Taken together, a reasonable suggestion would be that future studies on the development of autoimmune and age-related disorders should take both age and gender into account in order to be able to draw more accurate conclusions relevant to sex and age and thus contribute to personalized medicine. Moreover, clinical trials testing drugs for immune-mediated diseases should dedicate more resources to study specifically the more susceptible populations such as the elderly and, in some cases, like in autoimmune disorders, the female population.

Do age-related alterations in the immune system increase the risk of developing autoimmunity for patients undergoing cancer immunotherapy with immune checkpoint inhibitors?

The immune system of an elderly person is likely to demonstrate age-associated changes that may include alterations in signaling pathways that in turn may influence the function of effector and regulatory T cells increasing the likelihood of T cell aberrant behavior. Within the T cell compartment, such changes include reduced proliferative potential, accumulation of CD28-negative T cells, reduced TCR diversity and signal transduction, increased expression of inhibitory receptors (i.e. CTLA-4, PD-1 and LAG-3), reduced numbers of naïve cells (especially CD8), and increased numbers of memory T cells and regulatory T cells (8–10). In addition, expression and function of immune checkpoints may be altered due to the chronic, low-grade inflammation which potentially may lead to different responses to ICI treatment in the elderly compared to younger patients evaluated in clinical trials (11). Recently, our group demonstrated enhanced expression of PD-1 and other activation markers by CD4+ T cells of young but not old patients with metastatic melanoma.
In this study, circulating CD4+ T cells in young patients with metastatic melanoma were shown to be strongly activated in comparison to relatively dormant CD4+ T cells of old melanoma patients (12). Such subtle differences between young and old patients might contribute to unfavorable behavior regarding ICI treatment in the elderly. Further research is needed to understand the link between age-related cellular and molecular changes and their potential influence on DC and T cell pathways involved in immune responses leading to the development of cancer or autoimmunity and more specifically, in immune responses to ICI treatment. In a recent review, Daste and colleagues assessed safety and efficacy of ICI therapies in elderly patients from several clinical trials. Regarding safety, the extracted data showed that young and old patients had similar tolerance to the ICI therapy. Concerning efficacy, the results from the extracted data seemed to be different between young and elderly patients according to the type of cancer; in some cases less efficacy was reported in the elderly. However, the elderly population is underrepresented in most clinical trials and more research is needed (11).

The link between age-related alterations in the immune system and an increased risk of autoimmunity due to ICI therapy is not clear. However, with the increasing use of ICI treatment, the accompanying incidence of immunotoxicity and autoimmunity will have to be monitored closely especially in challenging populations such as patients with autoimmune disorders, immune-compromised patients and elderly individuals. This thesis provided information on the impact of age and sex on IC molecule expression by immune cells of healthy individuals showing that elderly women often present a decrease in PD-1+ CD4+ T cells. Since PD-1 blockade has been associated with increased Th1 and Th17 responses in cancer patients (13), a recommendation would be that IC therapy should be specifically tailored to individual patients in order to prevent autoimmunity especially in elderly women.

Could targeting VISTA be a new therapeutic strategy to treat age-related, auto-immune vasculitides?

Aberrant immune checkpoint expression has been reported in several auto-immune diseases with most studies focusing on the inhibitory PD-1/PD-L1 and CTLA-4 checkpoint pathways (14–16). For instance, in RA, the PD-1 pathway was found to be up-regulated in the synovium of patients with active RA and demonstrated to be able to regulate T cell responses (14). In SLE, decreased regulation of PD-1 expression and function was found to be the result of both a single-nucleotide polymorphism (SNP) in the PD1 gene (PD1.3) as well as the inflammatory environment characterized by high levels of inflammatory cytokines and complexes of self DNA with autoantibodies (15,17,18). Moreover, in MS, the PD-1/PD-L1 pathway was found
to play an important role in modulating disease activity as increased expression together with higher IL-10 production, lower proliferation and increased apoptosis of myelin basic protein-specific cells have been associated with disease remission in MS patients (16).

Particularly for vasculitides, data on immune checkpoint expression and function is limited. Two studies assessing the expression of CTLA-4 and PD-1 on T and B cells of GPA patients have documented increased expression of both inhibitory checkpoint molecules (19,20). Additional evidence pointing to a role for CTLA-4 in the regulation of GPA comes from a small, open-label, clinical trial conducted in non-severe relapsing GPA patients with abatacept, a fusion protein consisting of the ligand-binding domain of CTLA-4 coupled to a modified Fc domain derived from IgG1. In this study, the use of abatacept was associated with a higher frequency of disease remission and prednisone discontinuation (21). Moreover, Wilde and colleagues demonstrated that immune activation in GPA was associated with an increased expression of the negative immune checkpoint molecule PD-1 on circulating T cells (20). Interestingly, in the aforementioned study, assessment of PD1 expression in renal biopsies of GPA patients demonstrated that the majority of infiltrating T cells lacked expression of PD-1 suggesting that in the lesional microenvironment the PD1 pathway fails to suppress T cell responses (20). Furthermore, PD-1-mediated suppression of T cell activation in vitro was less potent using T cells from GPA patients than those from HCs despite increased expression levels of PD-1 (20).

In GCA the relationship between modulation of IC expression and the distribution of Th cell subsets has been demonstrated using a humanized model of vasculitis. In this model, PD-1 blockade enhanced vascular inflammation and tissue production of IFN-γ and IL-17, the signature cytokines of Th1 and Th17 cells respectively (22). In contrast, little is known about the role of VISTA in vasculitis. Regarding the participation of VISTA in innate immune responses, Li and colleagues employed an imiquimoid (IMQ)- induced murine model of psoriasis mediated by the IL-23/IL17 axis resembling human psoriasis (23). Topical application of IMQ, a TLR7 agonist, resulted in skin inflammation due to the stimulation of DCs and IL-17-producing γδ TCR+ T cells. Moreover, Li et al. demonstrated that VISTA deficiency intensified inflammatory responses of DCs, IL-17-producing γδ T cells and CD4+ Th17 T cells (23). The IL-23/IL-17 inflammatory axis is crucial for the development of autoimmune and inflammatory disorders (24). In active GPA patients IL-17 and IL-23 cytokines have been reported to be increased together with PR3-specific Th17 cells. These two cytokines participate in the early stages of the development of GPA pathogenesis. After the activation of antigen presenting cells (APCs) by peptidoglycan and superantigens from S. aureus, the production of IL-23 skews and maintains the Th17
cell phenotype while IL-17 itself promotes the release of pro-inflammatory cytokines TNF-α and IL-1β produced by macrophages (25). Of note, persistent activation of T cells, especially Th1 and Th17 cells, also occurs in GCA patients (26–30). In this thesis we provide further evidence for the role of VISTA in Th1, Th17 and Tfh lineage differentiation. Hence, involvement of VISTA regulating the IL-23/IL-17 inflammatory axis in both GPA and GCA is likely to occur.

I therefore speculate that the increased VISTA expression seen on immune cells of GPA patients might be in part responsible for the induction of remission in GPA through the suppression of pathogenic Th cell responses. In addition, VISTA could be involved in the development of autoantibody-mediated effects in GPA. Previously, our group reported increased frequencies of IL-21-producing BCL-6+ CD4+ T cells in peripheral blood of ANCA-positive GPA patients (31). Given that increased VISTA expression failed to control Tfh lineage differentiation in GCA, increased frequencies of VISTA positive cells in GPA patients in remission could play a role in controlling IL-21 expression by Tfh cells. Evidently, further investigation on the functional consequences of the increased expression of VISTA observed in GPA patients is warranted.

In GCA we are the first to demonstrate that VISTA is involved in disease pathogenesis. We demonstrated that decreased VISTA expression fails to control Th1, Th17 and Tfh lineage differentiation. This could mean that VISTA expression on the surface of these cells is insufficient to properly transmit the negative signal. This may also be true at the site of inflammation since vascular inflammation and occlusion appear to be ongoing processes despite the increase of VISTA-expressing cells in the infiltrated layers of the vascular wall. In the future, additional knowledge on the function of VISTA and other IC molecules could translate into therapeutic approaches aimed to target VISTA and/or other (negative) IC molecules to halt excessive immune activation in vasculitides and other (auto) immune-mediated diseases.

At present, the diagnostic tests and procedures used to enable an accurate diagnosis of vasculitis include blood and urine tests, imaging, angiography and biopsies. However, due to the subtle signs and symptoms presented by some patients, vasculitis continues to pose diagnostic and management challenges. A recommendation for addressing vasculitis is to use novel techniques for immune monitoring with multiple-angle approaches. For instance, combining information from tissue material and whole blood material using automated analysis might facilitate monitoring complex immune dynamics and the identification of novel subsets. This will allow the identification of disease state signatures and aid tailored care.

In particular for VISTA, one of the main IC molecules studied in this thesis, future research should focus on the validation of the binding partner of VISTA, VSIG-3, as proposed by Wang et. al (32). Further studies on the VSIG-3/VISTA pathway...
may unveil additional clues on how to modulate this pathway and could lead to a potential target for immunotherapy to control autoimmune disorders. Currently, monoclonal antibodies and small molecules targeting VISTA in combination with other negative IC pathways are being tested in early-stage clinical trials for cancer treatment (33). It is of prime importance to take advantage of the outcomes of such clinical trials to better understand the effects of VISTA-targeted therapies with respect to the inflammatory cell types affected and the entailed secreted soluble mediators as possible biomarkers present in blood to monitor immune response. Once this information is better comprehended, it will allow to exploit and mold the effects rendered by the VISTA pathway, either by inhibiting or enhancing T cell activation.

Figure 1. Schematic representation of immune checkpoint molecule involvement in vasculitides. The role of immune checkpoint (IC) molecules in vasculitides is emphasized by the appearance of immune related adverse events (irAEs) (i.e development of GCA) following immune checkpoint inhibitor (ICI) treatment. The immune system of Giant Cell Arteritis (GCA) patients is characterized by age-related changes such as altered intercellular communication and overall chronic low-grade inflammation. In this thesis, we demonstrated that there is decreased VISTA expression by CD4+ T cells which facilitates Th1, Th17 and Tfh lineage differentiation in GCA. Additional knowledge on the function of VISTA could translate in therapeutic approaches targeting negative immune checkpoint molecules to halt excessive immune activation seen in vasculitides.
CONCLUSION

Broadening our understanding of the multiple alterations endured by the immune system while ageing may aid in advancing our knowledge on the development and progression of age-associated autoimmune diseases. In my thesis I focused on alterations in surface protein expression, especially immune checkpoint molecules, on circulating immune cells of elderly individuals in health and disease. Among other findings, this thesis demonstrated that IC molecule expression by T cells is influenced by both age and sex. In addition, the involvement of IC in vasculitis was emphasized by the presented case report of GCA development following ICI treatment. Most interestingly, this thesis unveiled an important role for the negative IC VISTA in the immunopathology of large-vessel vasculitis. Further investigation on the role of age, gender and IC molecules is warranted to improve treatment options for patients suffering from vasculitides.
References


Summary, General Discussion and Future Perspectives


